

BC

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 38/00, 38/02, C07K 5/00, 7/00</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/21571</b>
			(43) International Publication Date: <b>6 May 1999 (06.05.99)</b>
(21) International Application Number: <b>PCT/US98/03298</b>		(81) Designated States: AU, CA, JP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: <b>20 February 1998 (20.02.98)</b>			
(30) Priority Data: <b>60/063,622 27 October 1997 (27.10.97) US</b>		<b>Published</b> <i>With international search report.</i>	
(71) Applicant: <b>TREGA BIOSCIENCES, INC. [US/US]; 3550 General Atomics Court, San Diego, CA 92121 (US).</b>			
(72) Inventors: <b>DOOLEY, Colette, T.; 844 Sapphire Street, San Diego, CA 92109 (US). GIRTEN, Beverly, E.; Apartment 111, 5220 Fiore Terrace, San Diego, CA 92122 (US). HOUGHTEN, Richard, A.; 4939 Rancho Viejo, Del Mar, CA 92014 (US).</b>			
(74) Agents: <b>PERKINS, Susan, M. et al.; Campbell &amp; Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).</b>			
<b>BEST AVAILABLE COPY</b>			
(54) Title: <b>MELANOCORTIN RECEPTOR LIGANDS AND METHODS OF USING SAME</b>			
(57) Abstract			
<p>The invention provides ligands for melanocortin receptors. For example, the invention provides the melanocortin receptor peptide ligand Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub>, where the iodo group is unlabeled or radioactively labeled. The invention additionally provides methods of assaying for melanocortin receptors in a cell or tissue such as brain. The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a melanocortin receptor ligand and to methods of administering the pharmaceutical composition to a subject. The invention further provides tetrapeptide ligands for melanocortin receptors and methods of altering melanocortin receptor activity.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

WO 99/21571

PCT/US98/03298

1

**MELANOCORTIN RECEPTOR LIGANDS AND METHODS OF USING SAME****BACKGROUND OF THE INVENTION****FIELD OF THE INVENTION**

The present invention relates generally to the  
5 fields of peptide chemistry and molecular pathology and  
more specifically to novel melanocortin receptor ligands.

**BACKGROUND INFORMATION**

The melanocortin (MC) receptors are a group of  
cell surface proteins that mediate a variety of  
10 physiological effects, including regulation of adrenal  
gland function such as production of the glucocorticoid  
cortisol and aldosterone; control of melanocyte growth  
and pigment production; thermoregulation;  
immunomodulation; and analgesia. Five distinct  
15 MC receptors have been cloned and are expressed in a  
variety of tissues, including melanocytes, adrenal  
cortex, brain, gut, placenta, skeletal muscle, lung,  
spleen, thymus, bone marrow, pituitary, gonads and  
adipose tissue (Tatro, Neuroimmunomodulation 3:259-284  
20 (1996)). Three MC receptors, MC1, MC3 and MC4, are  
expressed in brain tissue (Xia et al., Neuroreport  
6:2193-2196 (1995)).

A variety of ligands termed melanocortins  
function as agonists that stimulate the activity of  
25 MC receptors. The melanocortins include  
melanocyte-stimulating hormones (MSH) such as  $\alpha$ -MSH,  
 $\beta$ -MSH and  $\gamma$ -MSH, as well as adrenocorticotrophic hormone  
(ACTH). Individual ligands can bind to multiple

WO 99/21571

PCT/US98/03298

2

MC receptors with differing relative affinities. The variety of ligands and MC receptors with differential tissue-specific expression likely provides the molecular basis for the diverse physiological effects of melanocortins and MC receptors.

A particularly potent MC receptor ligand is an  $\alpha$ -MSH analogue, NDP. NDP has been used extensively to characterize MC receptors because it is chemically and enzymatically stable and binds with high affinity to all identified MC receptors. Despite the availability of NDP, no binding assay has been reported for the detection of MC receptors in brain tissue even though MC receptor messenger RNA is expressed in brain. Detection of MC receptors in brain is of particular interest since brain MC receptors mediate some of the physiological effects of melanocortins, including the antipyretic effect observed with experimentally induced fever.

Due to the varied physiological activities of MC receptors, high affinity ligands of MC receptors would be valuable to analyze the presence of MC receptors in particular cells or tissues. In addition, high affinity ligands of MC receptors could be used to exploit the varied physiological responses of MC receptors by functioning as potential therapeutic agents or as lead compounds for the development of therapeutic agents.

Thus, there exists a need for ligands that bind to MC receptors with high affinity and methods for detecting the presence of MC receptors in a cell or tissue such as brain. The present invention satisfies this need and provides related advantages as well.

WO 99/21571

PCT/US98/03298

3

### SUMMARY OF THE INVENTION

The invention provides ligands for MC receptors. For example, the invention provides the MC receptor peptide ligand Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub>, where the iodo group is unlabeled or radioactively labeled. The invention additionally provides methods of assaying for MC receptors in a cell or tissue such as brain. The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a melanocortin receptor ligand and to methods of administering the pharmaceutical composition to a subject. The invention further provides tetrapeptide ligands for MC receptors and methods of altering MC receptor activity.

15

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a saturation binding isotherm for <sup>125</sup>I-HP 467.

Figure 2 shows the association rate for <sup>125</sup>I-HP 467.

20

Figure 3 shows the dissociation rate for <sup>125</sup>I-HP 467.

Figure 4 shows a competition curve of unlabeled HP 467 for <sup>125</sup>I-HP 467.

Figure 5 shows an HP 467 saturation binding curve of mouse L cells expressing MC1.

Figure 6 shows an HP 467 saturation binding curve of mouse L cells expressing MC3.

WO 99/21571

PCT/US98/03298

4

Figure 7 shows an HP 467 saturation binding curve of mouse L cells expressing MC4.

Figure 8 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC1.

5        Figure 9 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC3.

Figure 10 shows a saturation binding isotherm for HP 467 on HEK 293 cells expressing MC4.

10       Figure 11 shows the percent bound for all library mixtures of a tetrapeptide scanning combinatorial library.

#### DETAILED DESCRIPTION OF THE INVENTION

The invention provides ligands for MC receptors and methods for detecting the presence of MC receptors in  
15 a cell or tissue. For example, the invention provides the MC receptor peptide ligand HP 467, having the amino acid sequence Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub> (SEQ ID NO:1). HP 467 is a para-iodinated form of HP 228 (see Table I), wherein the iodo group can be a  
20 stable nuclide such as <sup>127</sup>I or an unstable nuclide, for example, radioactive <sup>125</sup>I or <sup>131</sup>I. HP 228 is a heptapeptide analogue of NDP (see Table I) and, like NDP, is both more potent and more stable than α-MSH. Table I shows various MC receptor ligands.

WO 99/21571

PCT/US98/03298

5

**Table I. Melanocortin Receptor Ligands**


---

	$\alpha$ MSH:	Ac-SYSMEHFRWGKPV-NH <sub>2</sub>	(SEQ ID NO:2)
	NDP:	Ac-SYS (Nle) EHfRWGKPV-NH <sub>2</sub>	(SEQ ID NO:3)
5	$\gamma$ 1-MSH:	YVMGHFRWDRF-OH	(SEQ ID NO:4)
	$\gamma$ 2-MSH:	H <sub>2</sub> N-YVMGHFRWDRFG-OH	(SEQ ID NO:5)
	$\gamma$ 3-MSH:	H <sub>2</sub> N-YVMGHFRWDRFGRRNGSSSSGVGGAAQ-OH	(SEQ ID NO:6)
	ACTH <sub>(1-10)}</sub> :	MEHFRWG-OH	(SEQ ID NO:7)
10	$\beta$ -MSH:	H <sub>2</sub> N-AEKKDEGPYRMEHFRWGSPPKE-OH	(SEQ ID NO:8)
	HP 228:	Ac-(Nle)QHfRWG-NH <sub>2</sub>	(SEQ ID NO:9)

---

Amino acids provided throughout the application are identified by their well known one letter or three letter codes and as being in the D- or L-configuration by designations "D" or "L," respectively or, alternatively, using lower case letters to designate amino acids in the D-configuration. Where not specified, an amino acid can be in the D- or L- configuration but is more likely in the L-form.

The structural relationship between HP 467 and various MC receptor ligands suggested that HP 467 could function as an MC receptor ligand. As disclosed herein, HP 467 exhibits high affinity binding to MC receptors in rat brain homogenates and in cell lines transfected with various MC receptors (see Examples III and IV).

The invention also provides MC receptor tetrapeptide ligands. A combinatorial library was prepared by the positional scanning format (U.S. Patent No. 5,556,762, issued September 17, 1996, which is incorporated herein by reference) and screened to find

WO 99/21571

PCT/US98/03298

6

smaller ligands that bind to MC receptors. The present invention thus further provides tetrapeptide ligands that bind to MC receptors. Such tetrapeptides have the structure A1-B2-C3-D4, where "A1" is  $\alpha$ FmLys, L-hmP, His, L-Nal, Arg, D-Arg, e-Lys, Lys, D-pyrala, D-Lys, D-His, D-Ala, Thiopro, L-isoN or 3-2Met; "B2" is Arg, D-Thi, pCl-f, D-Phe, Arg,  $\alpha$ -Orn, pF-F, D-His, D-Lys, e-Lys,  $\delta$ -Orn, Thiopro, t4-benz, L-hmP or D-Cit; "C3" is Arg, L-Cha, D-Ile, D-Arg, pCl-F, D-Lys,  $\alpha$ -Orn, pCl-f, D-Ser, L-hmP, L-pyrala, D-His, Npecot, eAca, D-Cit or Thiopro; and "D4" is D-Nal, D-Arg, D-His, e-Lys, Lys, D-Lys or D-Glu. Abbreviations of amino acid derivatives used throughout the application are shown in Table II.

As disclosed herein, a positional scanning combinatorial library was constructed to contain  $91^4$  (68,574,960) tetrapeptides having the general structure A1-B2-C3-D4. In positional scanning libraries, a defined amino acid is determined for a given position and is "walked" through the length of the peptide, resulting in the defined amino acid appearing in positions A1, B2, C3 and D4. Mixtures active at each of the four positions can be identified in a single screen. The following peptides were synthesized: Ac-OXXX-NH<sub>2</sub>; Ac-XOXX-NH<sub>2</sub>; Ac-XXOX-NH<sub>2</sub>; and Ac-XXXO-NH<sub>2</sub>; where "O" is a defined single amino acid and "X" represents a mixture of 91 L-, D- and amino acid derivatives such that each peptide is represented as 91 mixtures each containing 753,571 peptides.



WO 99/21571

PCT/US98/03298

7

Table II. Abbreviations of Amino Acid Derivatives

	<u>Abbreviation</u>	<u>Full Name</u>
	e-Aca	e-aminocaproic acid
5	t4-benz	trans-4-(NHCH <sub>2</sub> )cyclohexyl-COOH
	Boc	t-butoxycarbonyl
	Cbz	benzyloxycarbonyl
	Cha	cyclohexylalanine
	Cit	citrulline
10	Fmoc	fluorenylmethoxycarbonyl
	αFmLys	lysine (α-Fmoc)
	hmp	homoproline
	isoN	isoasparagine
	e-Lys	αN-Cbz-lysine (Boc)
15	3-2Met	3-amino-2-methyl-propionic acid
	Nal	naphthylalanine
	Npecot	nipecotic acid
	α-Orn	ornithine (Cbz)
	δ-Orn	αN-Cbz-ornithine (Boc)
20	pCl-F	L-4-chlorophenylalanine
	pCl-f	D-4-chlorophenylalanine
	pF-F	L-4-fluorophenylalanine
	pyrala	(3-pyridyl)alanine
	Thi	(2-thienyl)alanine
25	Thioprop	thiopropine

Using a positional scanning combinatorial library, a number of high affinity tetrapeptide MC receptor ligands were identified (see Example X).  
 30 Particularly active peptides are provided herein having the structure A1-B2-C3-D4, where "A1" is αFmLys or His; "B2" is Arg, D-Thi, or pCl-f; "C3" is Arg, L-Cha, or

WO 99/21571

PCT/US98/03298

8

D-Ile; and "D4" is D-Nal or D-Arg (see Table V in Example X). Based on results with these peptides, additional peptides were synthesized with the following amino acids in specific positions in the tetrapeptide:

5  $\alpha$ FmLys and His at position "A1"; Arg, D-Thi and pCl-f at position "B2"; Arg, L-Cha and Ile at position "C3"; and D-Nal and D-Arg at position "D4". Synthesis of peptides containing these amino acids resulted in 36 individual tetrapeptides that were tested for activity. Several

10 peptides were found to have high affinity for MC receptors (see Table VI in Example X).

The invention also provides the peptides

His-(pCl-f)-Arg-(D-Nal) (SEQ ID NO:10);

His-(pCl-f)-(L-Cha)-(D-Arg) (SEQ ID NO:11);

15 ( $\alpha$ FmLys)-(pCl-f)-Arg-(Nal) (SEQ ID NO:12);

( $\alpha$ FmLys)-Arg-(L-Cha)-(Nal) (SEQ ID NO:13);

( $\alpha$ FmLys)-Arg-(L-Cha)-(D-Arg) (SEQ ID NO:14);

( $\alpha$ FmLys)-(D-Thi)-Arg-(Nal) (SEQ ID NO:15);

( $\alpha$ FmLys)-Arg-Arg-(Nal) (SEQ ID NO:16); and

20 His-(pCl-f)-Arg-(D-Nal) (SEQ ID NO:17). The amino terminus for any of the tetrapeptides disclosed herein can be modified by acetylation and the carboxy terminus can be modified by amidation. As disclosed herein, the peptide Ac-His-(pCl-f)-Arg-(D-Nal)-NH<sub>2</sub> (SEQ ID NO:18) is a

25 high affinity MC receptor ligand having an IC<sub>50</sub>, the inhibitory concentration at which 50% of binding is inhibited, of 18 nM.

MC receptor ligands such as the peptides disclosed herein can be synthesized using a modification

30 of the solid phase peptide synthesis method of Merrifield (J. Am. Chem. Soc. 85:2149 (1964), which is incorporated herein by reference) or can be synthesized using standard solution methods well known in the art (see, for example,

WO 99/21571

PCT/US98/03298

9

Bodanszky, M., Principles of Peptide Synthesis (Springer-Verlag, 1984), which is incorporated herein by reference). Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, CA) or using the manual peptide synthesis technique described by Houghten, Proc. Natl. Acad. Sci., USA 82:5131 (1985), which is incorporated herein by reference. For example, HP 467 was synthesized as described in Example I.

Peptides can be synthesized using amino acids or amino acid analogs, the active groups of which are protected as required using, for example, a t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (Fmoc) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical Co.; Advanced Chemtec) or synthesized using methods known in the art. Peptides synthesized using the solid phase method can be attached to resins including 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl and 4-(hydroxymethyl)phenoxy methyl-copoly(styrene-1% divinylbenzene) (Wang resin), all of which are commercially available, or to p-nitrobenzophenone oxime polymer (oxime resin), which can be synthesized as described by De Grado and Kaiser, J. Org. Chem. 47:3258 (1982), which is incorporated herein by reference.

The choice of amino acids or amino acid derivatives incorporated into the peptide will depend, in part, on the specific physical, chemical or biological characteristics required of the MC receptor peptide ligand. Such characteristics are determined, in part, by the route by which the MC receptor ligand will be

WO 99/21571

PCT/US98/03298

10

administered or the location in a subject to which the MC receptor ligand will be directed.

Selective modification of the reactive groups in a peptide also can impart desirable characteristics to an MC receptor ligand. Peptides can be manipulated while still attached to the resin to obtain N-terminal modified compounds such as an acetylated peptide or can be removed from the resin using hydrogen fluoride or an equivalent cleaving reagent, then modified. Compounds synthesized containing the C-terminal carboxy group (Wang resin) can be modified after cleavage from the resin or, in some cases, prior to solution phase synthesis. Methods for modifying the N-terminus or C-terminus of a peptide are well known in the art and include, for example, methods for acetylation of the N-terminus or methods for amidation of the C-terminus. Similarly, methods for modifying side chains of the amino acids or amino acid analogs are well known to those skilled in the art of peptide synthesis. The choice of modifications made to the reactive groups present on the peptide will be determined by the characteristics that are desired in the peptide.

A cyclic peptide also can be an effective MC receptor ligand. A cyclic peptide can be obtained by inducing the formation of a covalent bond between, for example, the amino group at the N-terminus of the peptide and the carboxyl group at the C-terminus. Alternatively, a cyclic peptide can be obtained by forming a covalent bond between a terminal reactive group and a reactive amino acid side chain or between two reactive amino acid side chains. The choice of a particular cyclic peptide is determined by the reactive groups present on the peptide as well as the desired characteristic of the

WO 99/21571

PCT/US98/03298

11

peptide. For example, a cyclic peptide can provide an MC receptor ligand with increased stability *in vivo*.

A newly synthesized peptide can be purified using a method such as reverse phase high performance liquid chromatography (RP-HPLC) as described in United States Patent No. 5,420,109, issued May 30, 1995, which is incorporated herein by reference. Alternatively, other methods of separation based on the size or charge of the peptide can be used for peptide purification. Furthermore, the purified peptide can be characterized using these and other well known methods such as amino acid analysis and mass spectrometry (see U.S. Patent No. 5,420,109).

The invention also relates to pharmaceutical compositions comprising an MC receptor ligand such as HP 467 and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize the MC receptor ligand or increase the absorption of the agent. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a

WO 99/21571

PCT/US98/03298

12

physiologically acceptable compound, depends, for example, on the route of administration of the MC receptor ligand and on the particular physico-chemical characteristics of the specific MC receptor ligand.

5           The invention further relates to methods of administering a pharmaceutical composition comprising an MC receptor ligand such as HP 467 to a subject in order to restrain pathologically elevated cytokine activity in the subject. For example, HP 467 can be administered to  
10 a subject as a treatment for inflammation, pain, cachexia and patho-immunogenic diseases such as rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus, each of which is characterized by pathologically elevated cytokine activity. As used  
15 herein, the term "pathologically elevated" means that a cytokine activity is elevated above a range of activities that is expected in a normal population of such subjects. For example, a normal range of IL-1 activity present in a specific tissue can be determined by sampling a  
20 statistically significant number of normal, healthy subjects in the population. A subject having a pathology characterized by cytokine-induced pathological effects can be identified by determining that the cytokine activity in the subject is pathologically elevated above  
25 the normal range. In particular, a pathologically elevated level of cytokine activity is at least about one standard deviation above the normal, and can be two standard deviations or greater above the normal range.

A pharmaceutical composition comprising an MC  
30 receptor ligand such as HP 467 can be administered to a subject having pathologically elevated cytokine activity by various routes including, for example, orally, intravaginally, rectally, or parenterally, such as

WO 99/21571

PCT/US98/03298

13

intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intracisternally or by passive or facilitated absorption through the skin using, for example, a skin patch or transdermal iontophoresis, respectively. Furthermore, the composition can be administered by injection, intubation or topically, the latter of which can be passive, for example, by direct application of an ointment or powder, or active, for example, using a nasal spray or inhalant. A cytokine restraining agent also can be administered as a topical spray, in which case one component of the composition is an appropriate propellant. The pharmaceutical composition also can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed., CRC Press, Boca Raton, FL (1993), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

The invention also provides methods of assaying for the presence of an MC receptor in a sample by contacting the sample with a radiolabeled peptide, for example,  $^{125}\text{I}$ -HP 467. The method further consists of removing unbound radiolabeled peptide from the sample and determining the binding of the radiolabeled peptide. In addition to labeling the peptide with a radioactive moiety, the peptide also can be modified to introduce a chemical moiety that can be readily detected, for example, a fluorescent moiety, as long as the introduced chemical moiety does not interfere with binding of the peptide to one or more MC receptors.

WO 99/21571

PCT/US98/03298

14

The sample is contacted with the radiolabeled peptide under conditions that allow specific binding of the peptide to the sample. One skilled in the art will know or can readily determine conditions that allow specific binding of the radiolabeled peptide to the sample. Such conditions include, for example, temperature, pH and time of incubation. For example, the conditions that allow specific binding of a peptide to a sample are generally about physiological pH, at a temperature between about 4°C and 37°C, and for a time of about 30 min to 16 hr. Such conditions include, for example, those disclosed in Examples III and IV.

Binding assays of MC receptor transfected cell lines as well as a receptor binding assay in rat brain homogenates have been developed. Initial experiments using a tritiated ligand failed to yield specific binding, so the disclosed assay of the invention was developed using <sup>125</sup>I-HP 467. Mouse L cells and human embryonic kidney (HEK) 293 cell lines were transfected with various MC receptors to determine if HP 467 displayed specificity for MC receptor types. Advantages of HEK 293 cells over L cells for MC receptor assays include the high receptor numbers per cell for all receptors including MC1, tight Scatchard plots for all receptors including MC1, and the human cell origin of the HEK 293 cell lines expressing human receptors, which can reflect MC receptor binding in human better than cell lines from other species.

Binding kinetics and competition with standard analogues confirmed that the binding site of HP 467 is an MC receptor (see Examples III and IV). Thus, the present invention provides a new radioligand for MC receptors,



WO 99/21571

PCT/US98/03298

15

Ac-Nle-Gln-His-(p(<sup>125</sup>I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub>  
(<sup>125</sup>I-HP 467).

The effects of HP 467 on cytokines, via its binding to MC receptors, are similar to those for HP 228 (see Examples V through IX). The effect of HP 228 on cytokines and the uses provided thereby are described, for example, in U.S. Patent No. 5,420,109, WO 95/13086 and WO 96/27386, each of which is incorporated herein by reference. The present invention provides a method of restraining a pathologically elevated cytokine activity in a subject by administering to the subject an effective amount of HP 467. The pathologically elevated cytokine activity can be due, for example, to inflammation, cachexia, or a patho-immunogenic disease. Interestingly, however, HP 467 antagonizes HP 228 induced hypophagia and acute metabolic effects caused by HP 228 (see Examples VIII and IX). Therefore, HP 467 can additionally be used to antagonize HP 228 in the areas of obesity (food intake) and metabolism. In addition, HP 467 can be used as a lead compound for new drug discovery related to antagonism of known MC agonists.

Cytokine expression can result in damage to healthy tissue in a subject and, in extreme cases, can lead to severe disability and death. Cytokines can be expressed at a site of localized infection or can be expressed systemically, for example, in an immune response or in response to bacterial endotoxin-induced sepsis. Cytokine expression can induce pyrexia (fever) and hyperalgesia (extreme sensitivity to pain) in a subject, as well as macrophage and monocyte activation, which produces or further contributes to an inflammatory response in a subject.

WO 99/21571

PCT/US98/03298

16

Since cytokine expression can be localized or systemic, one skilled in the art would select a particular route and method of administration of HP 467 based on the source and distribution of cytokines in a subject. For example, in a subject suffering from a systemic condition such as bacterial endotoxin-induced sepsis, a pharmaceutical composition comprising HP 467 can be administered intravenously, orally or by another method that distributes the compound systemically.

However, in a subject suffering from a pathology caused by localized cytokine expression such as acute respiratory distress syndrome, HP 467 can be suspended or dissolved in the appropriate pharmaceutically acceptable carrier and administered directly into the lungs using a nasal spray or other inhalation device.

In order to restrain the biological activity of a cytokine, HP 467 must be administered in an effective dose, which is about 0.01 to 100 mg/kg body weight. The total effective dose can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the concentration of HP 467 required to obtain an effective dose in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for restraining cytokine activity.

WO 99/21571

PCT/US98/03298

17

Examples of the effectiveness of HP 467 in preventing or minimizing adverse biological effects mediated by cytokines are provided in Examples V through IX and summarized in Table IV. HP 467 can effectively  
5 restrain cytokine expression in mice and provide relief from cytokine-mediated swelling and lethality. Thus, HP 467 can be used as a medicament for the treatment of pathologies such as inflammation, pain, cachexia and patho-immunogenic diseases such as arthritis,  
10 inflammatory bowel disease and systemic lupus erythematosus, which are characterized by altered cytokine activity.

The following examples are intended to illustrate but not limit the invention.

15

**EXAMPLE I**Synthesis of HP 467

This example provides a standard preparation of HP 467.

HP 467 was synthesized essentially as described  
20 in United States Patent No. 5,420,109. Briefly, 100 mg MBHA resin containing a t-Boc Gly derivative was added to a reaction vessel suitable for solid phase peptide synthesis (Houghten, Proc. Natl. Acad. Sci. USA 82:5131 (1985), which is incorporated herein by reference). The  
25 following conditions were used for peptide synthesis: coupling was performed in 6-fold excess in N,N-dimethylformamide (DMF) with 0.2 M N-hydroxybenzotriazole (HOBt) and 0.2 M N,N-diisopropylcarbodiimide (DIC) for 90 minutes;  
30 activation was performed with 5% diisopropylethylamine (DIEA) in methylene chloride (DCM) for three washes of

WO 99/21571

PCT/US98/03298

18

2 min; deprotection was performed with  
55% trifluoroacetic acid (TFA) for 30 min; washes were  
performed with DCM and isopropyl alcohol (IPA); the  
ninhydrin test was run after washing with DMF, DCM and  
5 methanol; acetylation was performed with acetylimidazole  
in 40-fold excess DCM for 4 hr; and cleavage was  
performed with hydrofluoric acid (HF) and anisole for 90  
min.

Peptide synthesis was carried out with the  
10 sequential steps of activation, coupling of amino acid,  
ninhydrin test, deprotection and washing, and the steps  
were repeated for addition of a new amino acid at each  
cycle. The amino acids were coupled in the order D-Trp,  
L-Arg, *p*-iodo-D-Phe, L-His, L-Gln and L-Nle. The peptide  
15 was acetylated and the DNP protecting group was removed  
from His using 2.5% thiophenol in DMF followed by removal  
of formyl protecting groups in 25% HF in dimethyl-  
sulphide. The peptide was cleaved from the resin and  
processed as described previously (United States Patent  
20 No. 5,420,109). The resulting peptide was approximately  
80 to 90% pure.

## EXAMPLE II

### Peptide Iodination

This example provides methods for iodinating a  
25 peptide.

For diazotization, 1.0 ml 2 N H<sub>2</sub>SO<sub>4</sub> was added to  
3.0 mg peptide containing *p*-NH<sub>2</sub>-Phe. A 6.67 µl aliquot  
(0.02 µmol) was transferred to a reaction vial, and  
79.3 µl of 2 N H<sub>2</sub>SO<sub>4</sub> was added. A 6.90 µl aliquot (0.02  
30 µmol) of 3 mM NaNO<sub>2</sub> was added, and the reaction was  
incubated at 0°C for 30-40 min.

WO 99/21571

PCT/US98/03298

19

For iodination, 100  $\mu$ l 2 N  $\text{H}_2\text{SO}_4$  and 400  $\mu$ l 0.5 M  $\text{CuSO}_4$  was added to 12.0 mg Zn powder, and the components were allowed to react with periodic mixing for 30-45 min, with venting, until bubbling stopped. The grains were washed twice with  $\text{H}_2\text{O}$ . For unlabeled peptide, 7.12  $\mu$ l of 0.67 mM NaI (0.0047  $\mu$ mol) was added to the reaction vial. For radiolabeled peptide, 0.0047  $\mu$ mol of  $\text{Na}^{125}\text{I}$  was added to the reaction vial. Approximately 1/8 of the copper grains was added to the vial, and the vial was vortexed 1 min. The reaction was carried out vented at room temperature for 3 hr with periodic mixing.

Samples were analyzed on a Vydac 218TP54 C-18 column and were monitored at 214 nm. Buffer A was 0.05% TFA in  $\text{H}_2\text{O}$ , and Buffer B was 0.05% TFA in acetonitrile. Samples were resolved using a 2% per minute gradient from 5 to 55% Buffer B in 25 min.

Using the method described in this example,  $^{125}\text{I}$ -HP 467 was routinely labeled to a specific activity of 2000 Ci/mmol. These results demonstrate that HP 467 can be iodinated to generate unlabeled iodo-peptide or high specific activity radiolabeled iodo-peptide.

### EXAMPLE III

#### Melanocortin Receptor Binding Assay Using $^{125}\text{I}$ -HP 467 In Rat Brain Homogenate

This example provides a binding assay using  $^{125}\text{I}$ -HP 467 to detect MC receptors in rat brain homogenate.

For assays, fine chemicals were obtained from Sigma (St. Louis MO) and GF/B plates and MICROSCINT were obtained from Packard Instrument Co. (Meriden CT).

WO 99/21571

PCT/US98/03298

20

Frozen rat brains were thawed and the thalamus and hypothalamus were dissected out and weighed. The tissue was homogenized in 40 ml buffer A (50 mM Tris-HCl, 2 mM EDTA, 10 mM CaCl<sub>2</sub>, 100  $\mu$ M PMSF, pH 7.4) in a Dounce  
5 homogenizer. The homogenate was centrifuged at 39,000 xg for 10 min, resuspended in 20 ml fresh buffer and recentrifuged. The pellet was resuspended in 80 ml buffer B (50 mM Tris-HCl, 2 mM EDTA, 10 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 100  $\mu$ M PMSF, pH 7.4). <sup>125</sup>I-HP 467 was custom  
10 labeled by Amersham to a specific activity of 2000 Ci/mmol (Amersham; Arlington Heights IL). Fifty pM <sup>125</sup>I-HP 467 and 0.2 mg protein/ml membrane suspension containing 2 mg/ml BSA was added to each assay tube.

Typical assay volumes were 50  $\mu$ l <sup>125</sup>I-HP 467,  
15 50  $\mu$ l HP 228 and 250  $\mu$ l membrane. Competition assays were performed using  $\alpha$ -MSH,  $\gamma$ 1-MSH,  $\gamma$ 2-MSH,  $\gamma$ 3-MSH and ACTH (4-10) also containing 1 mM phenanthroline, 200  $\mu$ g/ml bacitracin, and 5  $\mu$ g/ml leupeptin. Tubes were incubated for 2 hours at 37°C. The assay was terminated  
20 by filtration through GF/B filters previously soaked in 5 mg/ml BSA Tris-HCl buffer. The samples were washed with Tris-HCl, dried and counted in Packard Minaxl gamma counter (Packard Instrument Co.).

Binding was found to be tissue specific with  
25 most binding observed in rat hypothalamus. As shown in Figures 1 to 4, binding of <sup>125</sup>I-HP 467 was saturable. Saturation binding curves of <sup>125</sup>I-HP 467 bound to rat brain membranes indicated a Kd of 0.4 nM and a Bmax of 21 fmoles/mg protein.

30 These results demonstrate that <sup>125</sup>I-HP 467 can be used in a binding assay to detect MC receptors in rat brain homogenates.

WO 99/21571

PCT/US98/03298

21

**EXAMPLE IV****Melanocortin Receptor Binding Assay Using  $^{125}\text{I}$ -HP 467 in Transfected Cells**

This example demonstrates the use of

5  $^{125}\text{I}$ -HP 467 for assaying MC receptor binding in human and mouse cell lines transfected to express MC receptors.

All cell culture media and reagents were obtained from GibcoBRL (Gaithersburg MD), except for COSMIC CALF SERUM (HyClone; Logan UT). HEK 293 and mouse

10 L cell lines were transfected with the human MC receptors hMC1, hMC3, and hMC4 (Gantz et al., Biochem. Biophys. Res. Comm. 200:1214-1220 (1994); Gantz et al., J. Biol. Chem. 268:8246-8250 (1993); Gantz et al. J. Biol. Chem. 268:15174-15179 (1993); Haskell-Leuvano et al., Biochem.

15 Biophys. Res. Comm. 204:1137-1142 (1994); each of which is incorporated herein by reference). Vectors for construction of an hMC5 expressing cell line were obtained, and a line of HEK 293 cells expressing hMC5 was constructed (Gantz, *supra*, 1994). hMC5 has been

20 described previously (Franberg et al., Biochem. Biophys. Res. Commun. 236:489-492 (1997); Chowdhary et al., Cytogenet. Cell Genet. 68:1-2 (1995); Chowdhary et al., Cytogenet. Cell Genet. 68:79-81 (1995), each of which is incorporated herein by reference). L cell lines were

25 maintained in MEM containing 25 mM HEPES, sodium pyruvate, 10% COSMIC CALF SERUM, 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.2 mg/ml G418 to maintain selection. For HEK 293 cells, DMEM was used instead of MEM, and 2 mM glutamine, non-essential amino acids, and

30 vitamins were included in addition to the above mentioned additives.

WO 99/21571

PCT/US98/03298

22

Before assaying, cells were washed once with phosphate buffered saline ("PBS"; without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), and stripped from the flasks using 0.25% trypsin and 0.5 mM EDTA. Cells were suspended in PBS, 10% COSMIC CALF SERUM and 1 mM  $\text{CaCl}_2$ . Cell suspensions were prepared at a density of  $2 \times 10^4$  cells/ml for HEK 293 cells expressing hMC3, hMC4 or hMC5, and  $1 \times 10^5$  cells/ml for HEK 293 cells expressing hMC1. For L cells, cells expressing MC3 or MC4 were suspended at  $2 \times 10^5$  cells/ml, and cells expressing MC1 suspended at  $8 \times 10^5$  cells/ml. Suspensions were placed in a water bath and allowed to warm to  $37^\circ\text{C}$  for 1 hour.

Binding assays were performed in a total volume of 250  $\mu\text{l}$  for HEK 293 cells, and a volume of 600  $\mu\text{l}$  for L cells. Peptides and other compounds were dissolved in distilled water.  $^{125}\text{I}$ -HP 467 (2000 Ci/mmol) was prepared in 50 mM Tris, pH 7.4, 2 mg/ml BSA, 10 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , 2 mM EDTA and added to each tube, with 50,000 dpm for HEK 293 assays or 100,000 dpm for L cell assays. To each tube was added  $4 \times 10^3$  HEK 293 cells expressing hMC3, hMC4 or hMC5, or  $2 \times 10^4$  cells expressing hMC1. For L cells expressing hMC3 or hMC4,  $1 \times 10^5$  cells were used, and for L cells expressing hMC1,  $4 \times 10^5$  cells were used. Assays were incubated for 2.5 hr at  $37^\circ\text{C}$ .

GF/B filter plates were prepared by soaking for at least one hour in 5 mg/ml BSA and 10 mM  $\text{CaCl}_2$ . Assays were filtered using a Brandel 96-well cell harvester (Brandel Inc.; Gaithersburg, MD). The filters were washed four times with cold 50 mM Tris, pH 7.4, the filter plates were dehydrated for 2 hr and 35  $\mu\text{l}$  of MICROSCINT was added to each well. Filter plates were counted using a Packard Topcount (Packard Instrument Co.) and data analyzed using GraphPad PRISM v2.0 (GraphPad



WO 99/21571

PCT/US98/03298

23

Software Inc.; San Deigo CA) and Microsoft EXCEL v5.0a (Microsoft Corp.; Redmond WA).

Binding assays were performed in duplicate in a 96 well format utilizing a 1.2 ml cluster tube system (Corning Costar; Cambridge MA). HP 467 was prepared in 50 mM Tris, pH 7.4, and  $^{125}\text{I}$ -HP 467 was diluted to give 100,000 dpm per 50  $\mu\text{l}$ . Serial dilutions (10-fold or 5-fold) of HP 228,  $\alpha$ -MSH, NDP and unlabeled HP 467 were prepared from 1.2 mM stock solutions. All assay tubes contained 50  $\mu\text{l}$  of  $^{125}\text{I}$ -HP 467 and 50  $\mu\text{l}$  of either Tris buffer (for determination of total binding) or diluted peptide (serial dilutions of HP 228,  $\alpha$ -MSH, NDP or unlabeled HP 467).

Saturation binding experiments were performed in duplicate in the same volume with the same number of cells as described above for the binding assay. Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations of the radioligand. Scatchard analysis of the data was used to determine receptor number and ligand affinity.  $^{125}\text{I}$ -HP 467 was added to the assay, with  $2 \times 10^8$  dpm being the highest concentration and 2-fold serial dilutions made thereafter to obtain 8 to 12 data points. Nonspecific binding was determined in the presence of unlabeled HP 467 at 1000 times the concentration of the  $^{125}\text{I}$ -HP 467. The actual amount of tracer added was determined by gamma counting the test tubes on a Packard Minaxl gamma counter. The concentration of  $^{125}\text{I}$ -HP 467 in the assay was calculated from the half-life corrected dpm using EXCEL v5.0a.

The results of displacement binding assays using L cells for MC receptors hMC1, hMC3, and hMC4 are

WO 99/21571

PCT/US98/03298

24

summarized in Table III. Saturation curves for the L cells are shown as Figures 5, 6 and 7 for MC1, MC3 and MC4, respectively. The  $K_i$  apparent and displacement binding assays determined from receptor binding assays for hMC5 in HEK 293 cells also are included in Table III. Saturation binding isotherms and Scatchard plots for MC1, MC3 and MC4 in HEK 293 cells are included as Figures 8, 9 and 10, respectively. BMAX is indicated as receptors/cell and KD is nM.

As shown in Figures 5 through 10,  $^{125}\text{I}$ -HP 467 can bind to the MC receptors MC1, MC3 and MC4. As shown in Table III,  $^{125}\text{I}$ -HP 467 also binds to the MC5 receptor. The binding of  $^{125}\text{I}$ -HP 467 can be displaced by various MC receptor ligands with differing  $\text{IC}_{50}$  values depending on the specific MC receptor expressed in the cell line.

WO 99/21571

PCT/US98/03298

25

TABLE III - DISPLACEMENT OF $^{125}\text{I}$ -HP 467					
PEPTIDE		RECEPTOR			
		MC-1	MC-3	MC-4	MC-5
HP 228	Binding $\text{IC}_{50}$	1.49	29.70	7.51	194.50
	S. Dev.	0.78	5.23	2.95	78.49
	n	3	4	4	2
HP 467	Binding $\text{IC}_{50}$	0.23	0.48	0.31	0.46
	S. Dev.	0.17	0.25	0.20	0.25
	n	11	15	14	6
$\alpha$ -MSH	Binding $\text{IC}_{50}$	9.31	21.52	98.12	
	S. Dev.	6.41	7.92	30.24	
	n	4	6	5	0
NDP	Binding $\text{IC}_{50}$	1.90	2.01	4.36	5.85
	S. Dev.	1.44	1.53	2.79	1.92
	n	5	5	6	2

"S. Dev." is the Standard Deviation; "n" represents the number of samples.

MC1, MC3 and MC4 receptors were expressed in mouse

L cells and MC5 in HEK 293 cells. Concentrations ranged from  $10^{-5}$  to  $10^{-14}$  M.

#### EXAMPLE V

##### Reduction of Lipopolysaccharide-Induced Tumor Necrosis Factor Levels in Mice

This example describes the effectiveness of HP 467 for decreasing tumor necrosis factor (TNF) levels in lipopolysaccharide (LPS; endotoxin) treated mice.

BALB/c female mice weighing approximately 20 g were placed into a control group and three HP 467.

WO 99/21571

PCT/US98/03298

26

treatment groups. Five mg/kg of LPS in 0.9% saline was administered by intraperitoneal (IP) injection to all mice. Mice in the treatment group received either 30, 150 or 300 µg of HP 467 per mouse in a volume of 100 µl.

5 Control mice received 100 µl of saline alone. One minute after initial injections all mice received the LPS injection.

Blood samples were collected from the orbital sinus of treated and control mice 90 minutes after LPS administration. The plasma was separated by centrifugation at 3000 x g for 5 min and stored at -20°C. Samples were thawed and diluted with four volumes of 1x PBS (pH 7.4) containing 1% bovine serum albumin. A 100 µl sample of plasma was assayed by ELISA for TNF-α (Genzyme; Cambridge MA).

The mean ( $\pm$  SEM) TNF-α level in five mice from each group was determined and the percent reduction in TNF-α levels was calculated. As shown in Table IV, treatment of mice with HP 467 decreased the levels of TNF-α in a dose dependent manner when compared to saline controls with a 3% decrease observed with 30 µg/mouse, a 78% decrease with 150 µg/mouse and an 81% decrease with 300 µg/mouse.

These results indicate that HP 467 can restrain LPS-induced cytokine activity.

WO 99/21571

PCT/US98/03298

27

TABLE IV - BIOLOGICAL DATA FOR HP 467

5	<u>Biological Test</u>	<u>Dose</u>	<u>Efficacy</u>
	Reduction in TNF levels	30 µg/mouse	3%
		150 µg/mouse	78%
		300 µg/mouse	81%
	Inhibition of LPS-Induced	300 µg/mouse BID	50%
10	lethality	500 µg/mouse TID	40%
	Reduction in arachidonic	100µg/mouse	82%
	acid-induced ear swelling		
	<u>Antagonism of HP 228</u>		
		<u>Drug/Dose</u>	<u>Change</u>
15			<u>Day 1</u> <u>Day 2</u>
	Hypophagia	Saline	+5.3%    +12.1%
	(food intake)	HP 228 (1.5 mg/Kg)	-6.8%    -5.3%
		HP 228/HP 467	+2.1%    +4.8%
	Oxygen Consumption	HP 228/HP 467	50% of HP 228
20		(5 mg/kg / 10 mg/kg)	

**EXAMPLE VI**Lipopolysaccharide-Induced Lethality

This example describes the effectiveness HP 467 in reducing lethality from sepsis induced by administration of LPS.

WO 99/21571

PCT/US98/03298

28

These experiments were performed based on information reported by Rivier et al., Endocrinology 125:2800-2805 (1989), which is incorporated herein by reference. Adult female BALB/c mice were provided food and water *ad libitum*. Mice were treated either IP every 8 hours (three times a day; TID) for 40 hours with 500 µg HP 467 in 200 µl saline or at 8 AM and 4 PM (twice a day; BID) for 40 hours with 300 µg of HP 467 in 200 µl saline. Control animals received injections of saline at the respective times of HP 467 treatment. Immediately following the first injection, 0.6 mg LPS endotoxin in 200 µl saline was administered to each mouse. All groups contained 10 mice.

As shown in Table IV, administration of HP 467 in both dosing regimens significantly increased survival as compared to the saline control mice. There was a 50% survival rate with the BID treatment regimen using HP 467 (300 µg/mouse) and a 40% survival rate with the TID treatment regiment using HP 467 (500 µg/mouse). All mice in the saline control group died within the 40 hours of the study (100% mortality).

These results show that HP 467 significantly inhibits LPS-induced lethality.

#### EXAMPLE VII

##### Reduction of Arachidonic Acid-Induced Ear Swelling in Mice

This example demonstrates that HP 467 can reduce arachidonic acid-induced ear swelling in mice.

Experiments were performed using female BALB/c mice weighing 18 to 23 grams. Saline or 100 µg HP 467

WO 99/21571

PCT/US98/03298

29

was administered IP, 30 minutes prior to topical application of arachidonic acid (Calbiochem-Novabiochem; San Diego CA). A 10  $\mu$ l pipette was used to apply 10  $\mu$ l of a 100 mg/ml arachidonic acid solution in acetone to the inner and outer surfaces of the right ear of each mouse. Ten ml of acetone (alone) was applied to the inner and outer surface of the left ear of each mouse.

Ear thickness was measured with a hand held spring loaded caliper 60 minutes after arachidonic acid application. Increase in ear thickness was calculated by subtracting the thickness of the control ear from the thickness of the arachidonic acid-treated ear. The value for each group is the average of the swelling observed in the mice of each group. The percent reduction in swelling is based on the swelling observed in the saline control group. As shown in Table IV, HP 467 reduced the level of arachidonic acid-induced ear swelling by 81%.

These results show that HP 467 significantly reduces arachidonic acid-induced ear swelling.

20

#### EXAMPLE VIII

##### HP 467 Antagonism of HP 228 Induced Hypophagia

HP 228 reduces body weight (see WO 96/27386). This example shows that HP 467 antagonizes the effect of HP 228-induced hypophagia, thereby reversing the undesirable decrease in food consumption that occurs following HP 228 administration.

Male Sprague-Dawley rats weighing 250 to 300 grams were divided into three treatment groups of 8 rats each. The control group received IP saline injections BID (1 ml/kg) at 8 AM and 4 PM. Two groups of rats

WO 99/21571

PCT/US98/03298

30

received HP 228 injections IP (1.5 mg/kg) at the same dosing schedule as the control group. After each injection, each rat immediately received a second injection. The second injection for the saline group and one HP 228 group was another injection of saline (1 ml/kg), and the second injection for the remaining HP 228 group was HP 467 at a dose of 3.0 mg/kg. All injections started at 4 PM on day 0 and ended on the afternoon of day 2. Food consumption and body weight measurements were taken daily, each morning.

As shown in Table IV, food intake in the saline control group increased daily as expected. HP 228 treatment caused a decrease in food consumption during the study period, and HP 467 reduced the decrease in food consumption caused by administration of HP 228. These results demonstrate that HP 467 antagonizes the effect of HP 228-induced hypophagia.

#### EXAMPLE IX

##### HP 467 Antagonism of Acute Metabolic Effects by HP 228

Weight loss observed with HP 228 correlates to an increased metabolic rate as determined by increased resting oxygen consumption (see WO 96/27386). This example shows that HP 467 antagonizes the acute metabolic effects caused by HP 228 administration.

Male Sprague-Dawley rats (250 to 350 grams) were divided into three treatment groups for the measurement of acute metabolic effects. All injections were administered IP. PBS (1 ml/kg) was administered to two of the groups. The third group received 10 mg/kg HP 467. Fifteen minutes after the initial injections, one PBS group received another injection of PBS (1 ml/kg)



WO 99/21571

PCT/US98/03298

31

and the second group received 5 mg/kg HP 228. The group receiving the initial injection of HP 467 received 5 mg/kg HP 228. Immediately after the second injection the rats were placed into the Oxymax System (Columbus Instruments; Columbus OH) for monitoring. Data was collected 10 minutes after the animal was acclimated to the cage and continued for a total of 50 minutes. The parameters measured were resting oxygen consumption ( $VO_2$ ), resting respiratory quotient (RQ), and total locomotor activity. As shown in Table IV, HP 467 attenuated the effects of HP 228 on  $VO_2$ .

These results demonstrate that HP 467 antagonizes the effect of HP 228 on food intake and oxygen consumption.

15

**EXAMPLE X****Tetrapeptide Ligands for Melanocortin Receptors**

This example demonstrates the use of a positional scanning combinatorial library to identify tetrapeptide ligands for MC receptors.

20

A positional scanning combinatorial library was constructed to contain  $91^4$  (68,574,960) tetrapeptides of the structure A1-B2-C3-D4. In positional scanning libraries, a defined amino acid is determined for a given position and is "walked" through the length of the peptide, resulting in the defined amino acid appearing in positions A1, B2, C3 and D4. Mixtures active at each of the four positions can be identified in a single screen. The following peptides were synthesized:

30

Ac-OXXX-NH<sub>2</sub>; 91 mixtures each containing 753,571 peptides;  
Ac-XOXX-NH<sub>2</sub>; 91 mixtures each containing 753,571 peptides;  
Ac-XXOX-NH<sub>2</sub>; 91 mixtures each containing 753,571 peptides;

WO 99/21571

PCT/US98/03298

32

Ac-XXXO-NH<sub>2</sub>; 91 mixtures each containing 753,571 peptides; where "O" is a defined single amino acid and "X" represents a mixture of 91 L-, D- and amino acids derivatives.

- 5           Figure 11 shows the percent bound for all library mixtures prepared from the positional scanning combinatorial library. Table V shows the IC<sub>50</sub> for the most active mixtures from the screening data. IC<sub>50</sub> values were determined in brain tissue as described in
- 10 Example III.

- Based on the results shown in Table V, peptides were synthesized with the following amino acids in specific positions in the tetrapeptide: αFmLys and His at position A1; Arg, D-Thi and pCl-f at position B2; Arg,
- 15 L-Cha and Ile at position C3; and D-Nal and D-Arg at position D4. Synthesis of peptides containing these amino acids resulted in  $2 \times 3 \times 3 \times 2 = 36$  individual tetrapeptides.

WO 99/21571

PCT/US98/03298

33

5

10

15

Table Va - IC <sub>50</sub> values for most active mixtures from the screening data					
Library No.	OXXX	IC <sub>50</sub> $\mu$ M	Library No.	XOXX	IC <sub>50</sub> $\mu$ M
73	$\alpha$ FmLys	88	106	R	14
83	L-hmP	118	168	D-Thi	224
7	H	122	160	pCl-f	235
55	L-Nal	155	116	f	238
15	R	158	125	r	281
34	r	203	141	$\alpha$ -Orn	283
49	e-Lys	214	161	pF-F	335
9	K	220	117	h	363
75	D-pyrala	256	119	k	377
28	k	271	140	e-Lys	538
26	h	304	142	$\delta$ -Orn	563
21	a	467	158	Thioprop	1457
67	Thioprop	549	177	t4-benz	3634
80	L-ison	765	174	L-hmP	6845
89	3-2Met	2753	182	D-Cit	NA

WO 99/21571

PCT/US98/03298

34

5

10

15

20

Table Vb - IC <sub>50</sub> values for most active mixtures from the screening data					
Library No.	XXOX	IC <sub>50</sub> $\mu$ M	Library No.	XXXO	IC <sub>50</sub> $\mu$ M
197	R	41	328	D-Nal	107
240	L-Cha	59	307	r	135
209	I	105	299	h	209
216	r	142	322	e-Lys	245
250	pCl-F	164	282	K	546
210	k	190	301	k	559
232	$\alpha$ -Orn	266	297	e	NA
251	pCl-f	648			
217	s	928			
265	L-hmP	1021			
256	L-pyrala	1216			
208	h	1847			
272	Npecot	2213			
229	eAca	3363			
273	D-Cit	NA			
249	Thioprop	NA			

The 36 peptides were screened for MC receptor binding activity in rat brain and in mouse B16 cells. The IC<sub>50</sub> values for some of these peptides are shown in Table VI. The peptide Ac-His-(pCl-f)-Arg-(D-Nal)-NH<sub>2</sub> was active below 1  $\mu$ M with an IC<sub>50</sub> value of 18 nM.

WO 99/21571

PCT/US98/03298

35

**Table VI. Activity of Tetrapeptides on Rat Brain and Mouse B16 Cells**

	Peptide	Rat Brain IC <sub>50</sub> (nM)	Mouse B16 IC <sub>50</sub> (nM)
5	Ac-H-(pCl-f)-R-(Nal)-NH <sub>2</sub>	18	68
	Ac-H-(pCl-f)-(L-Cha)-r-NH <sub>2</sub>	>5000	156
	Ac-(αFmLys)-(pCl-f)-R-(Nal)-NH <sub>2</sub>	427	41
	Ac-(αFmLys)-R-(L-Cha)-(Nal)-NH <sub>2</sub>	>4000	1143
	Ac-(αFmLys)-R-(L-Cha)-r-NH <sub>2</sub>	427	185
10	Ac-(αFmLys)-(D-Thi)-R-(Nal)-NH <sub>2</sub>	469	>4000
	Ac-(αFmLys)-R-R-(Nal)-NH <sub>2</sub>	1995	1416
	Ac-H-(pCl-f)-R-(D-Nal)-NH <sub>2</sub>	51	19

These results show that tetrapeptides that bind to MC receptors were identified from a positional scanning combinatorial library.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WO 99/21571

PCT/US98/03298

36

We claim:

1. A peptide, comprising the sequence:

Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub>.

2. A composition of matter, comprising a  
5 peptide and a pharmaceutically acceptable carrier, said  
peptide comprising the sequence:

Ac-Nle-Gln-His-(p(I)-D-Phe)-Arg-(D-Trp)-Gly-NH<sub>2</sub>.

3. A radiolabeled peptide, comprising the  
sequence:

10 Ac-Nle-Gln-His-(p(<sup>125</sup>I)-D-Phe)-Arg-(D-Trp)-  
Gly-NH<sub>2</sub>.

4. A method of assaying for the presence of a  
melanocortin receptor in a sample, comprising:

(a) contacting the sample with the  
15 radiolabeled peptide of claim 3;

(b) removing unbound radiolabeled peptide from  
said sample; and

(c) determining the binding of said  
radiolabeled peptide.

20 5. The method of claim 4, wherein said sample  
is a cell.

6. The method of claim 4, wherein said sample  
is a tissue homogenate.

WO 99/21571

PCT/US98/03298

37

7. The method of claim 6, wherein said tissue is brain.

8. A method of restraining a pathologically elevated cytokine activity in a subject, comprising  
5 administering to the subject an effective amount of the peptide of claim 1.

9. The method of claim 8, wherein said pathologically elevated cytokine activity is due to inflammation.

10 10. The method of claim 8, wherein said pathologically elevated cytokine activity is due to cachexia.

11. The method of claim 8, wherein said pathologically elevated cytokine activity is due to a  
15 patho-immunogenic disease.

12. A peptide, comprising the sequence:

A1-B2-C3-D4, wherein

A1 is  $\alpha$ FmLys, L-hmP, His, L-Nal, Arg, D-Arg, e-Lys, Lys, D-pyr ala, D-Lys, D-His, D-Ala, Thiopro,  
20 L-isoN or 3-2Met;

B2 is Arg, D-Thi, pCl-f, D-Phe, Arg,  $\alpha$ -Orn, pF-F, D-His, D-Lys, e-Lys,  $\delta$ -Orn, Thiopro, t4-benz, L-hmP or D-Cit;

C3 is Arg, L-Cha, D-Ile, D-Arg, pCl-F, D-Lys,  
25  $\alpha$ -Orn, pCl-f, D-Ser, L-hmP, L-pyr ala, D-His, Npecot, eAca, D-Cit or Thiopro; and

WO 99/21571

PCT/US98/03298

38

D4 is D-Nal, D-Arg, D-His, e-Lys, Lys, D-Lys or D-Glu.

13. The peptide of claim 12, wherein the amino terminus is modified by acetylation.

5 14. The peptide of claim 12, wherein the carboxy terminus is modified by amidation.

15. The peptide of claim 12, wherein

A1 is  $\alpha$ FmLys or His;

B2 is Arg, D-Thi, or pCl-f;

10 C3 is Arg, L-Cha, or D-Ile; and

D4 is D-Nal or D-Arg.

16. The peptide of claim 15, wherein the amino terminus is modified by acetylation.

15 17. The peptide of claim 15, wherein the carboxy terminus is modified by amidation.

18. The peptide of claim 15, having the structure selected from the group consisting of:

His-(pCl-f)-(L-Cha)-(D-Arg);

( $\alpha$ FmLys)-(pCl-f)-Arg-(Nal);

20 ( $\alpha$ FmLys)-Arg-(L-Cha)-(Nal);

( $\alpha$ FmLys)-Arg-(L-Cha)-(D-Arg);

( $\alpha$ FmLys)-(D-Thi)-Arg-(Nal);

( $\alpha$ FmLys)-Arg-Arg-(Nal); and

His-(pCl-f)-Arg-(D-Nal).

25 19. The peptide of claim 18, wherein the amino terminus is modified by acetylation.



WO 99/21571

PCT/US98/03298

39

20. The peptide of claim 18, wherein the carboxy terminus is modified by amidation.

21. The peptide of claim 15, having the structure His-(pCl-f)-Arg-(D-Nal).

5 22. The peptide of claim 21, wherein the amino terminus is modified by acetylation.

23. The peptide of claim 21, wherein the carboxy terminus is modified by amidation.

10 24. A composition of matter, comprising said peptide of claim 12 and a pharmaceutically acceptable carrier.

15 25. A method of altering the activity of a melanocortin receptor in a subject, comprising administering to the subject an effective amount of the peptide of claim 12.

WO 99/21571

PCT/US98/03298

1 / 12

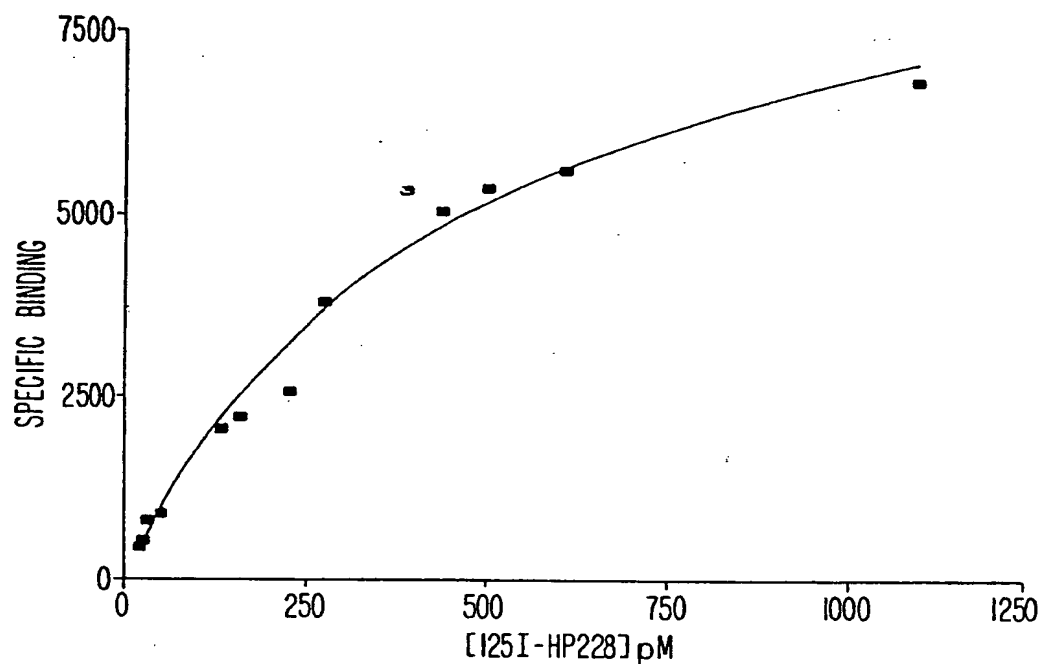


FIG. 1A

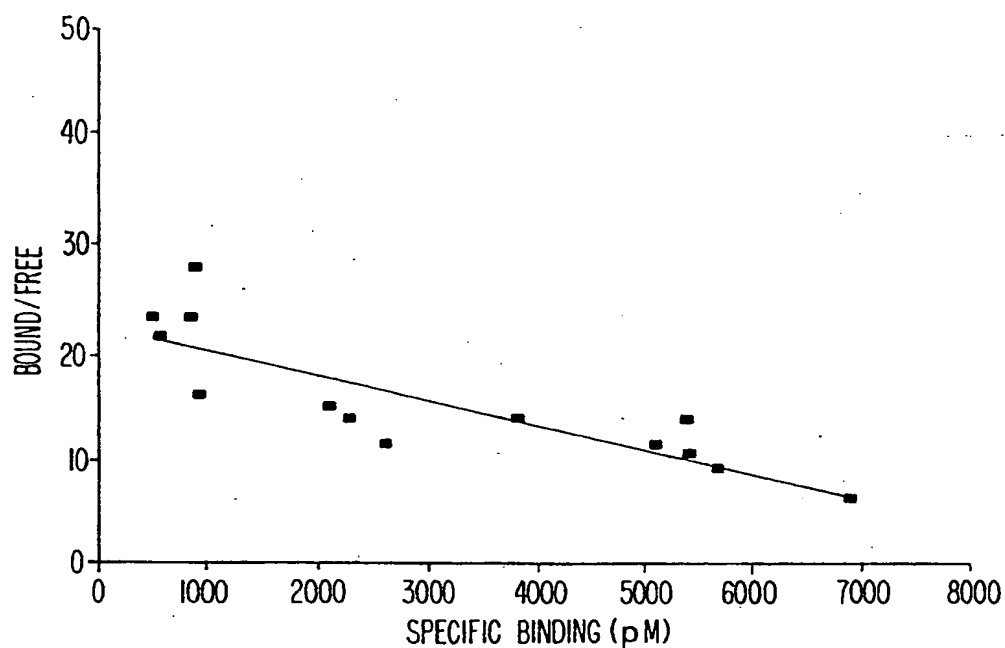


FIG. 1B

WO 99/21571

PCT/US98/03298

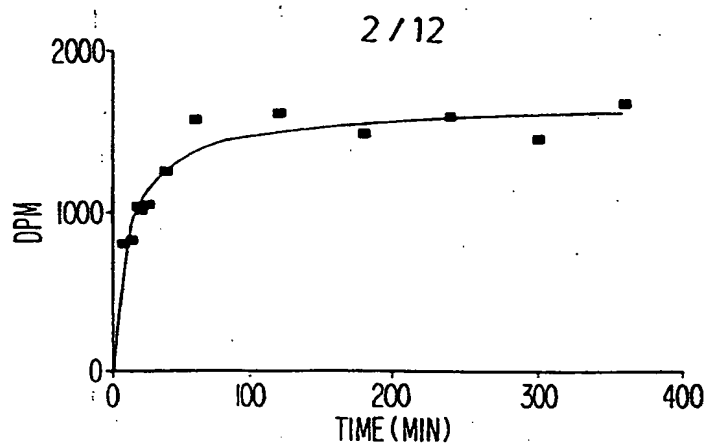


FIG. 2

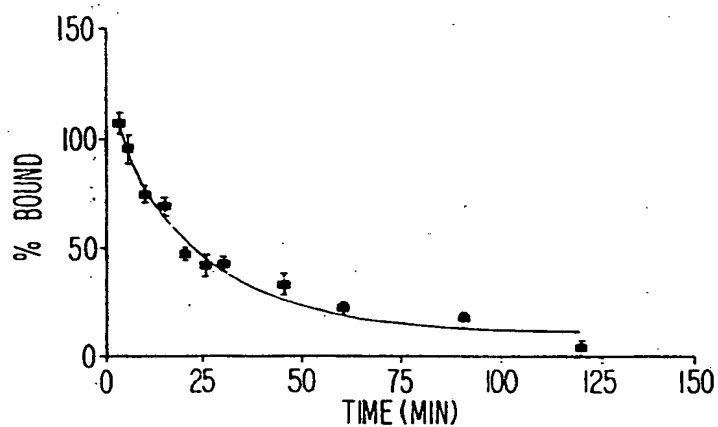


FIG. 3

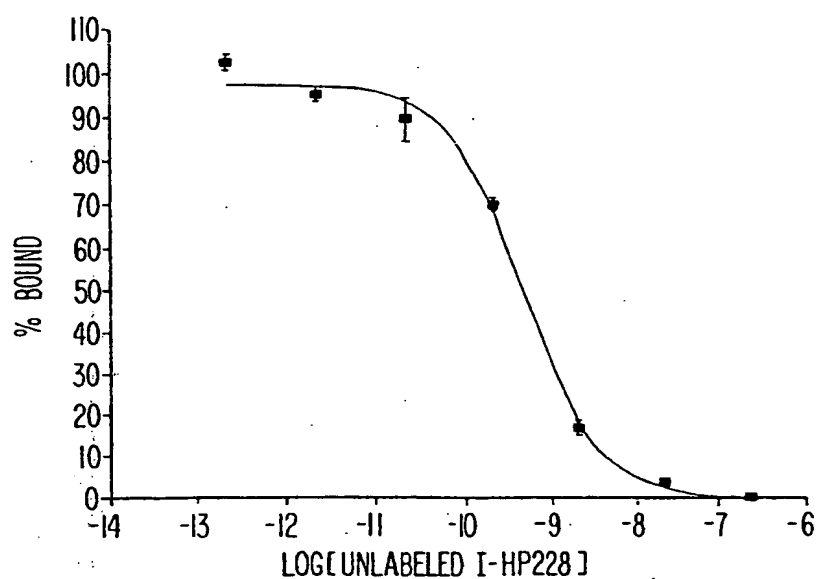


FIG. 4

WO 99/21571

PCT/US98/03298

3/12

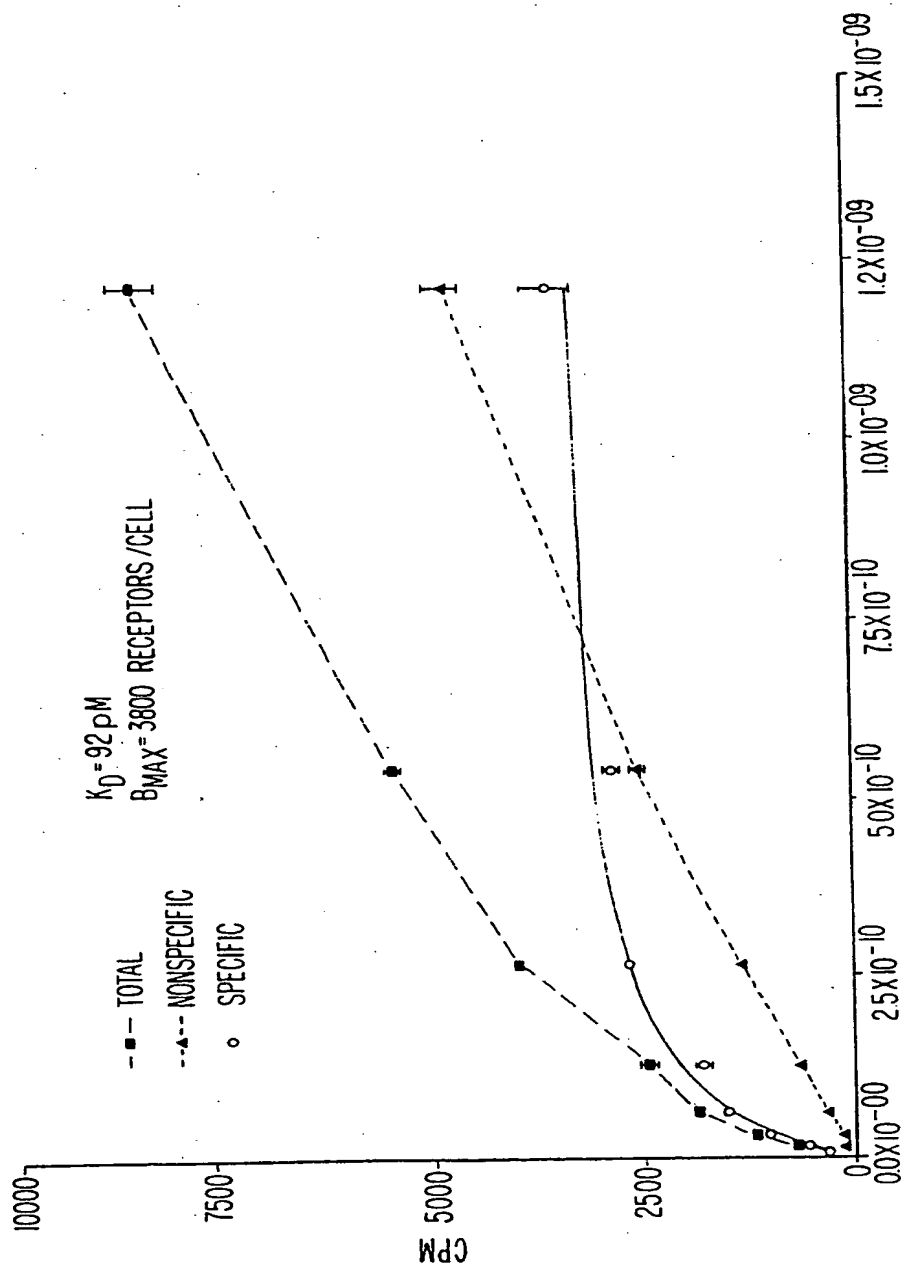


FIG.5

WO 99/21571

PCT/US98/03298

4/12

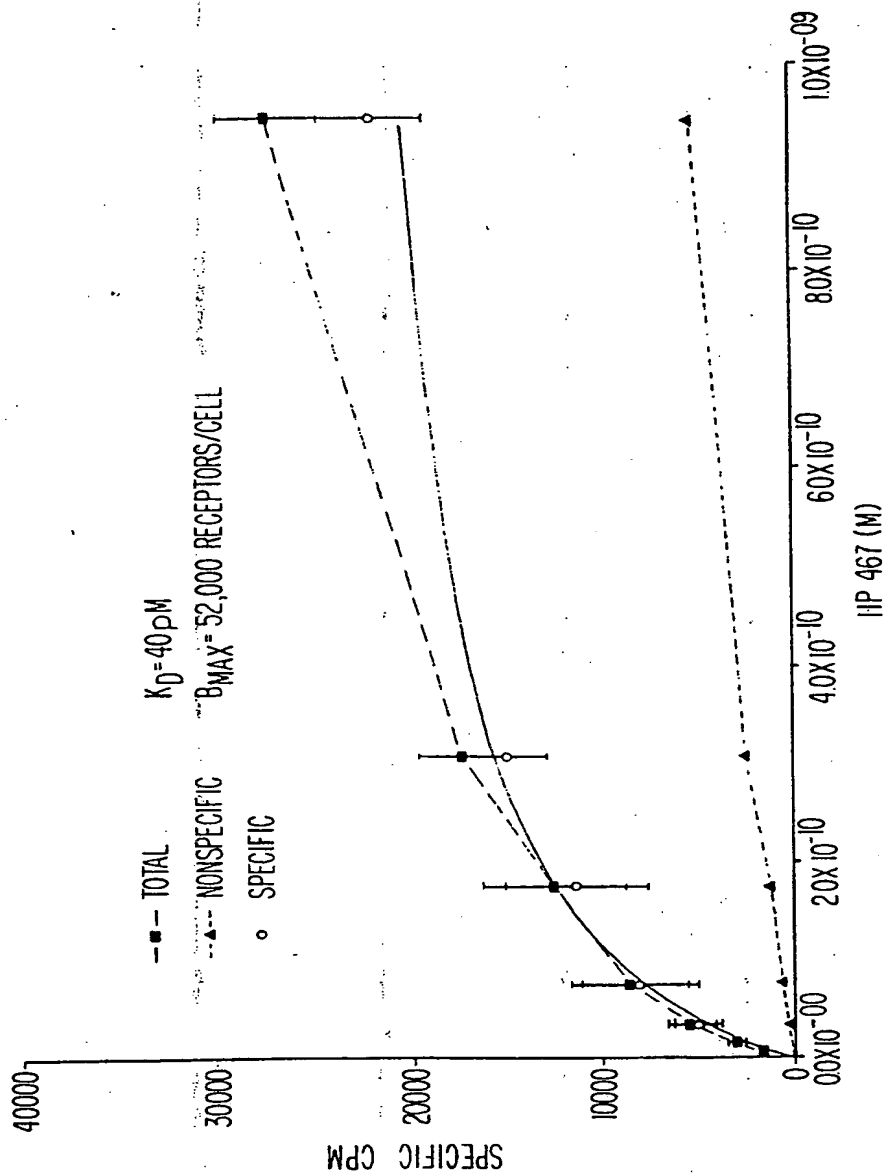


FIG.6

WO 99/21571

PCT/US98/03298

5/12

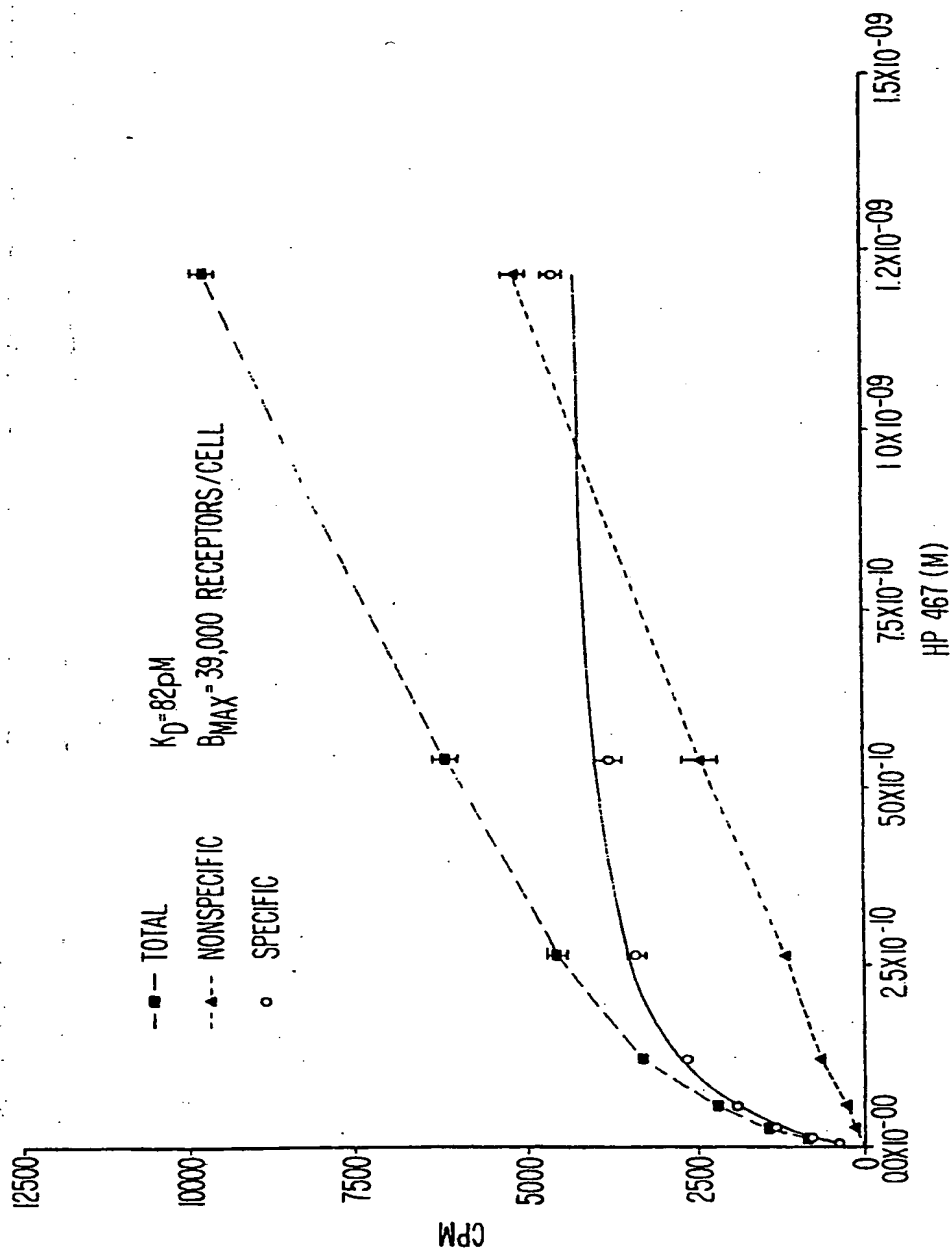


FIG. 7

WO 99/21571

PCT/US98/03298

6/12

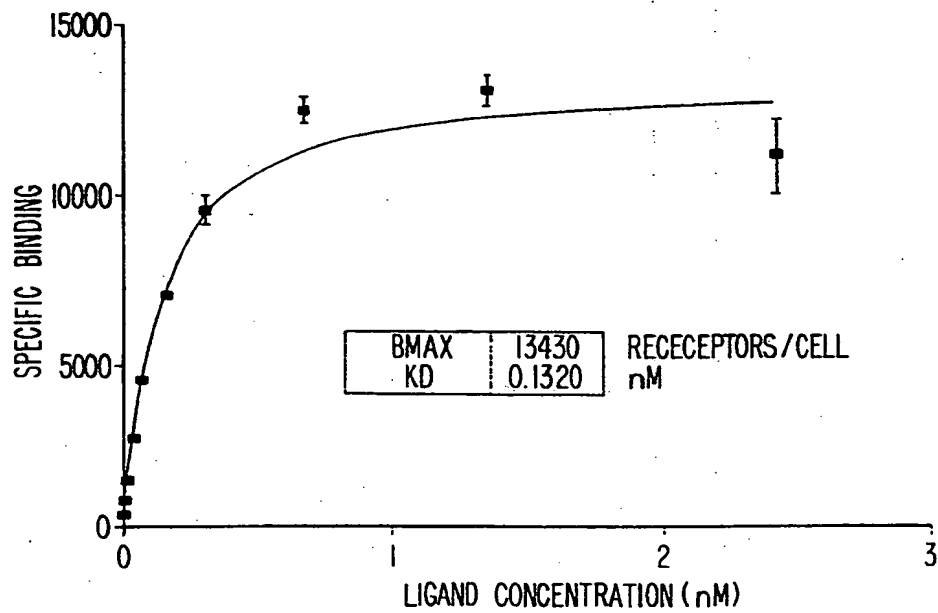


FIG. 8A

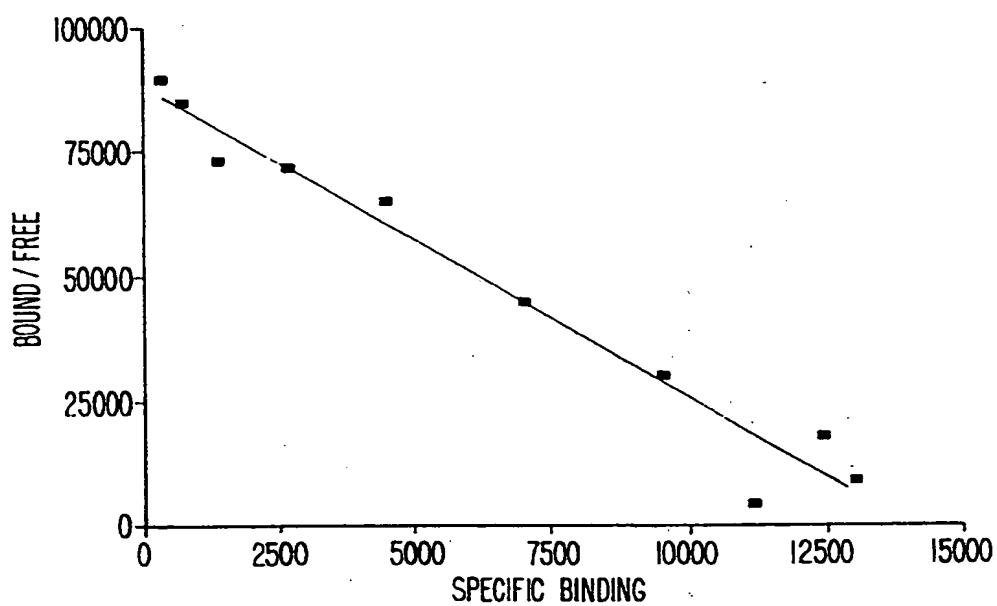


FIG. 8B

WO 99/21571

PCT/US98/03298

7/12

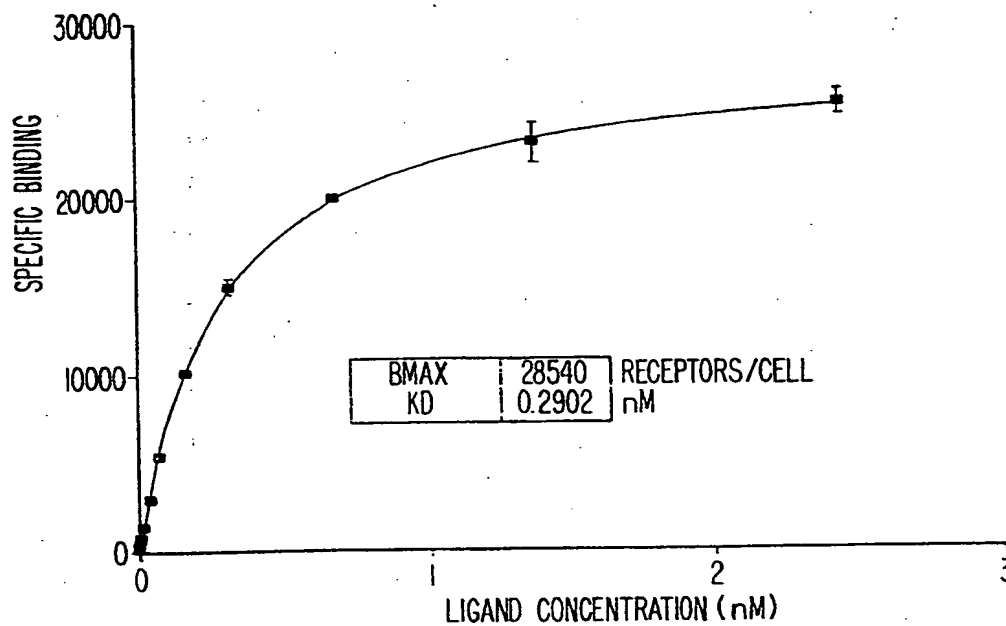


FIG. 9A

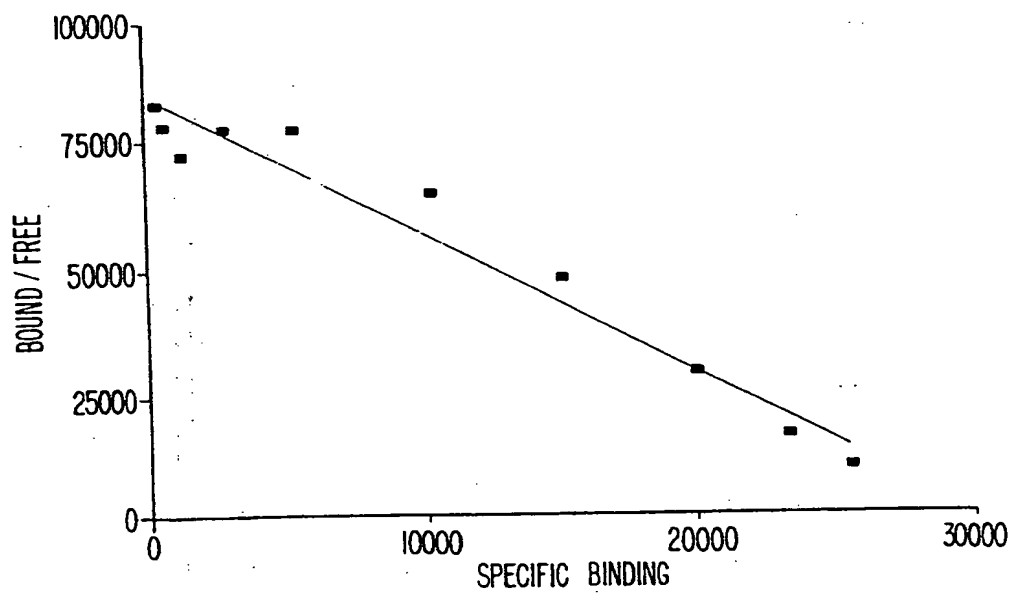


FIG. 9B



WO 99/21571

PCT/US98/03298

8/12

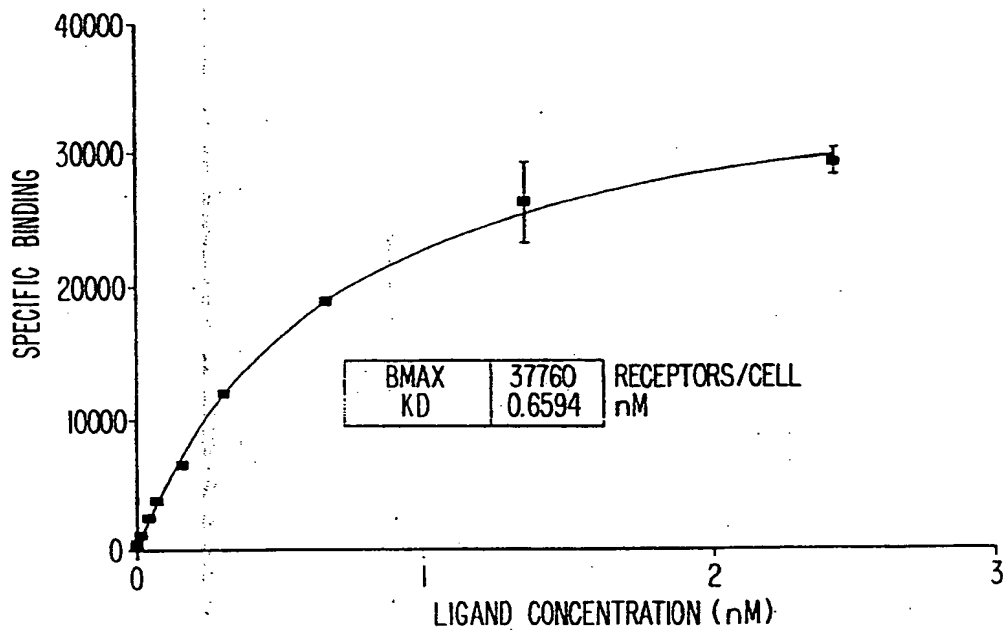


FIG. 10A

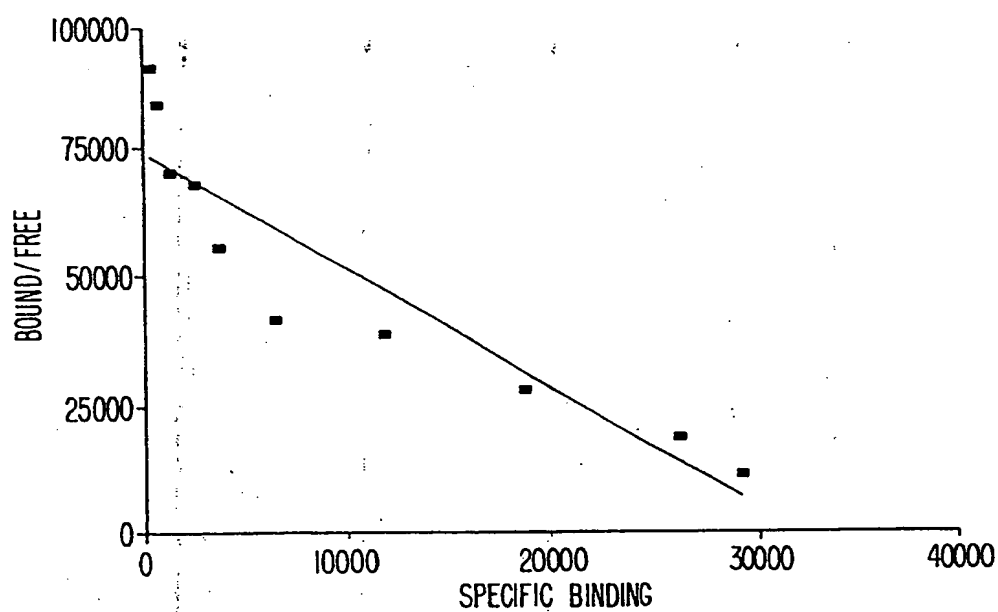


FIG. 10B

WO 99/21571

PCT/US98/03298

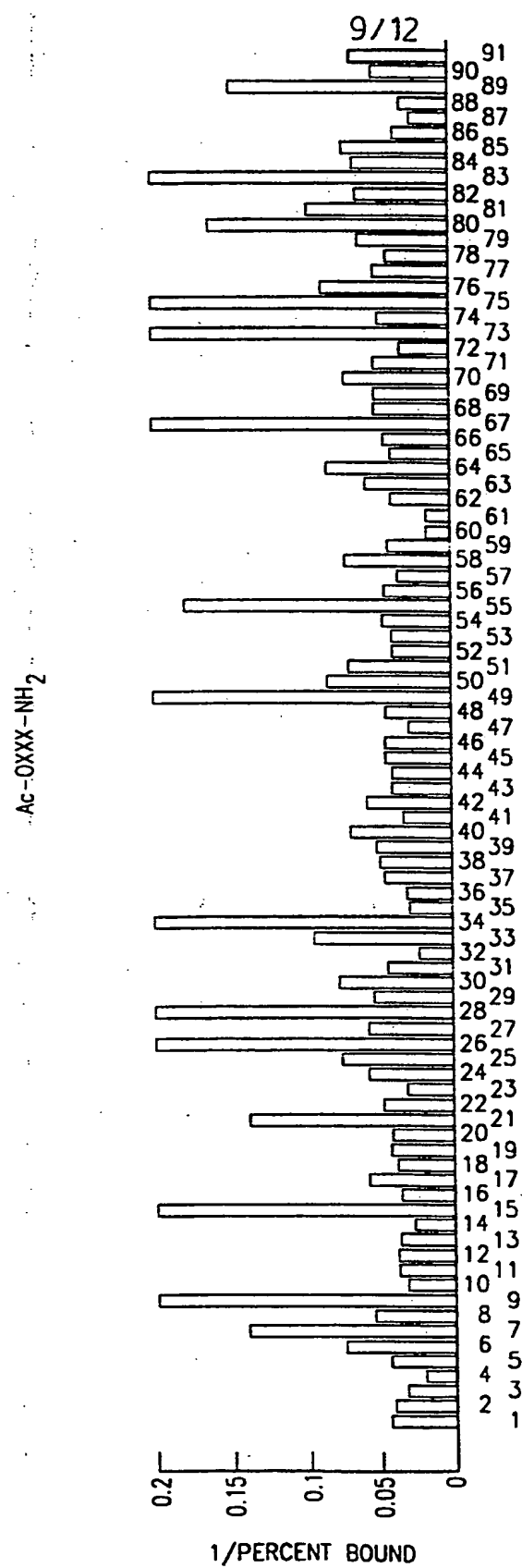


FIG. 11A

WO 99/21571

PCT/US98/03298

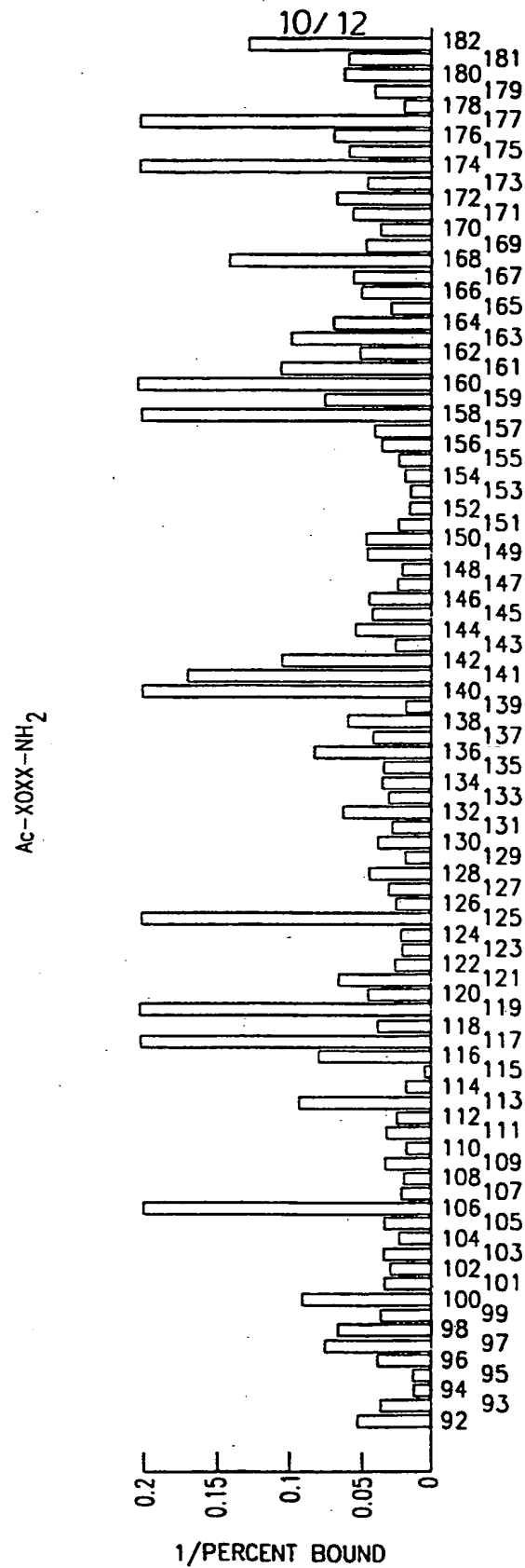


FIG. 11B

WO 99/21571

PCT/US98/03298

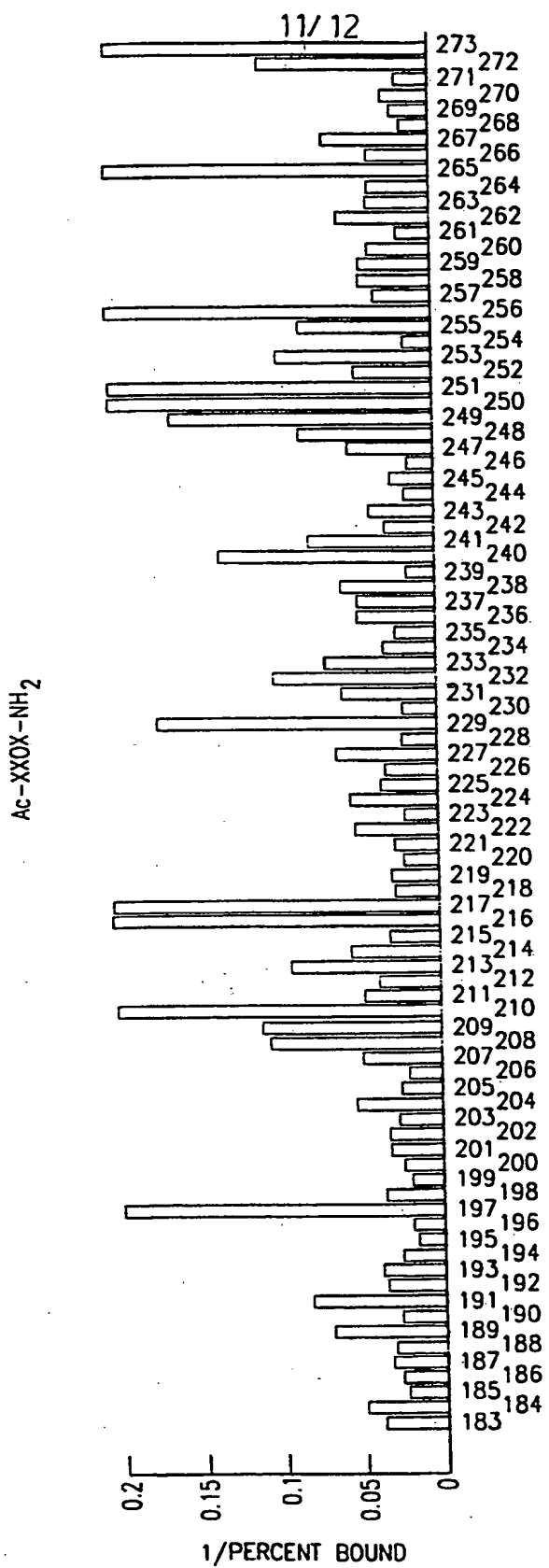


FIG. 11C

WO 99/21571

PCT/US98/03298

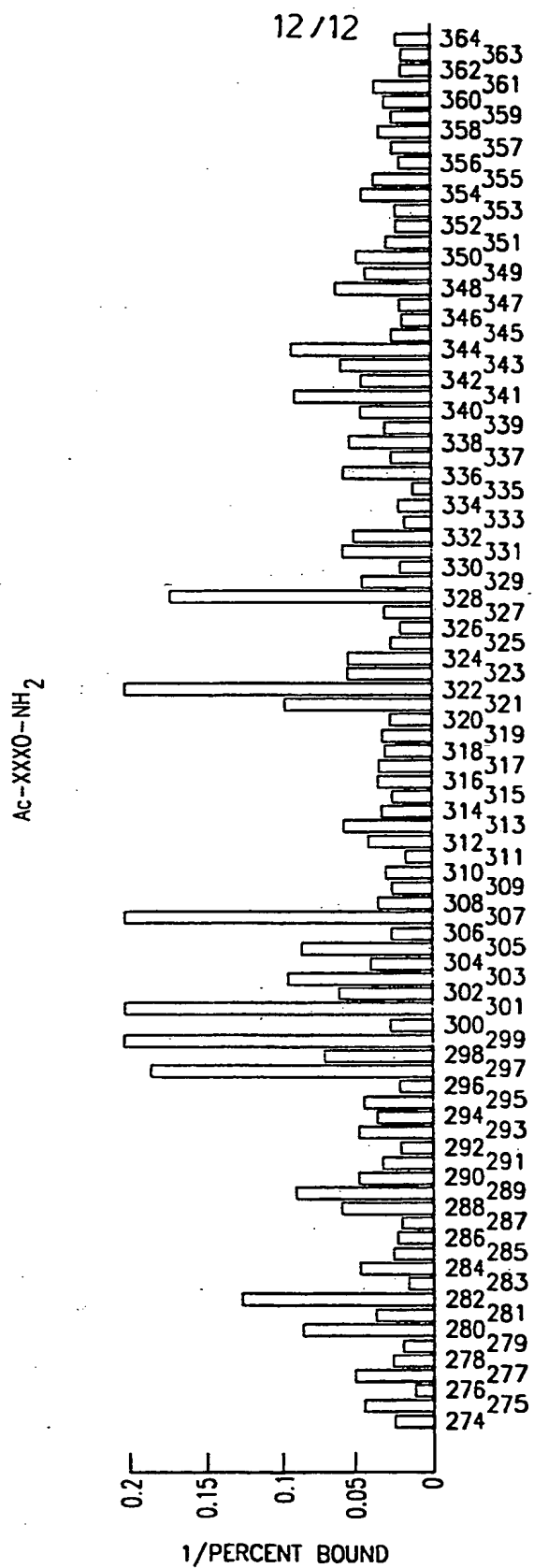


FIG. 11D

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/03298

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00, 38/02; C07K 5/00, 7/00

US CL : 530/329, 330; 514/16, 17, 18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/329, 330; 514/16, 17, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

LIBRARY CAS ONLINE, APS, BIOSIS, EMBASE, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,726,156 A (GIRTEN et al.) 10 March 1998, see entire document.	1-25
Y	US 5,618,791 A (DU) 08 April 1997, see entire document.	1-25
Y	WO 97/22356 A1 (HOUGHTEN PHARMACETICALS, INC.) 26 June 1997, see entire document.	1-25
Y	HASKELL-LUEVANO ET AL., Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R. J. Med. Chem., 1997, Vol. 40, No. 4, pages 2133-2139, see entire document.	1-25



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

03 AUGUST 1998

Date of mailing of the international search report

08SEP1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

*Doretha Lawrence For*  
AVIS M. DAVENPORT

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/03298

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHIOTH et al., Characterization of the binding of MSH-B, HP-228, GHRP-6 and 153N-6 to the human melanocortin receptor subtypes. Neuropeptides. 1997, Vol. 31, No. 6, pages 565-571, see entire document.	1-25

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**